

Purification and Characterization of a Proteinase Inhibitor from Field Bean, *Dolichos lablab perpureus* L.

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A proteinase inhibitor resembling Bowman-Birk family inhibitors has been purified from the seeds of cultivar HA-3 of *Dolichos lablab perpureus* L. The protein was apparently homogeneous as judged by SDS-PAGE, PAGE, IEF, and immunodiffusion. The inhibitor had 12 mole% 1/2-cystine and a few aromatic amino acids, and lacks tryptophan. Field bean proteinase inhibitor (FBPI) exhibited a pI of 4.3 and an M_r of 18,500 Da. CD spectral studies showed random coiled secondary structure. Conformational changes were detected in the FBPI-trypsin/chymotrypsin complexes by difference spectral studies. Apparent K_a values of complexes of inhibitor with trypsin and chymotrypsin were $2.1 \times 10^7 \text{ M}^{-1}$ and $3.1 \times 10^7 \text{ M}^{-1}$, respectively. The binary and ternary complexes of FBPI with trypsin and chymotrypsin have been isolated indicating 1:1 stoichiometry with independent sites for cognate enzymes. Amino acid modification studies showed lysine and tyrosine at the reactive sites of FBPI for trypsin and chymotrypsin, respectively.

KEY WORDS: *Dolichos lablab perpureus* L.; trypsin inhibitor; chymotrypsin inhibitor; purification; characterization; secondary structure; specificity; amino acid modification.

1. INTRODUCTION

Protein proteinase inhibitors are a class of proteins which form reversible, stoichiometric complexes with different classes of proteinases and competitively inhibit the catalytic activity of these enzymes. Multiple molecular forms of these proteins have been characterized from microbial, plant, and animal systems. These proteins have been studied for a long time as antinutritional factors. They have been implicated to have various physiological functions such as regulators of proteolytic cascades, safe storage proteins, and defense molecules against plant pests and pathogens (Ryan, 1990). They have also been used as cancer chemopreventive agents (Kennady, 1994). They have been isolated and characterized from a wide variety of plants, most notably from legumes (Laskowski and Kato, 1980; Moosor *et al.*, 1984; Norton, 1991; and references cited therein). Pro-

teinase inhibitors have been classified into two major classes, the pH and thermostable Bowman-Birk inhibitors (BBIs), which have M_r in the range of 8–10 kDa with high cysteine, and thermolabile Kunitz inhibitors, which have M_r in the range of 18–21 kDa. Further, serine proteinase inhibitors are designated as double-headed or single-headed inhibitors. Trypsin inhibitors are also identified as arginine- or lysine-type inhibitors, depending on the amino acid that contributes the carbonyl moiety of the scissile peptide bond.

The physicochemical properties along with the primary structure of proteinase inhibitors from different sources are useful in understanding the evolutionary process in different lines of organisms (Weder, 1985). They have also helped to establish the phylogenetic relationship among species/varieties of legume and nonlegume plants. Molecular and kinetic properties of the proteinase inhibitor isolated from a new cultivar, HA-3, of *Dolichos lablab perpureus* L. are described herein.

Dolichos lablab perpureus L., the field bean, is widely distributed in the tropics. It is a popular pulse crop whose pods are also consumed as a vegetable. The

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proteinase inhibitors in the seeds of the lablab bean were identified in the early 1940s. Banerji and Sohani (1969) reported partial purification of a proteinase inhibitor from a wild variety. However, the molecular properties of the proteinase inhibitor from this species have not been well described. Therefore, the present investigation was undertaken to purify the inhibitor from the newly developed cultivar, and study its molecular properties.

2. MATERIALS AND METHODS

2.1. Materials

Seeds of *D. lablab* were obtained from the Seed Processing Unit, University of Agricultural Sciences, GKVK, Bangalore, India.

N-Acetyl tyrosine ethyl ester (ATEE), QAE-Sephadex (Q-125, 120 μ), Sephadex G-75 (120–140 μ), *N*-benzoyl-D,L-arginine-*p*-nitroanilide-HCl (BAPNA), *N*-acetyl-phenylalanyl β -naphthyl ester, 5,5'-dithio-bis-(2-nitrobenzoic acid) [DTNB], molecular weight markers, 1-chloro-3-tosylamino-7-amino L-2 heptanone (TLCK), L-1-chloro-3-(4-tosylamide)-4-phenyl 2-butanone-HCl (TPCK), glucose oxidase, Freund's complete adjuvant, bovine trypsin (DCC treated), α -chymotrypsin (3 \times crystallized), 1,2-cyclohexane dione, *N*-acetyl imidazole, casein (technical grade), and 2,4,6-trinitrobenzene sulfonic acid (TNBS) were purchased from Sigma Chemical Co. (St. Louis, MO), and Pharmalyte pH 3–10 was purchased from Pharmacia (Upsala, Sweden). All other chemicals used were of analytical grade.

2.2. Methods

2.2.1. Assay of Trypsin and Chymotrypsin Inhibitory Activities

Caseinolytic activities of trypsin and chymotrypsin and their inhibition were measured according to the method of Kakade *et al.* (1970). One unit of enzyme is defined as 0.01 increase in the absorbance at 280 and 275 nm, respectively, for trypsin and chymotrypsin. The inhibitory units were number of trypsin/chymotrypsin units inhibited under the same conditions of assay.

Esterolytic activity of trypsin and pronase was assayed using BAPNA as substrate (Kakade *et al.*, 1969). Inhibitory assays were performed in a similar manner except with different aliquots of inhibitor. Esterolytic activity of chymotrypsin, subtilisin-A, and subtilisin-BPN were assayed using ATEE as substrate according to the method of Schwert and Takenaka (1955). The enzyme

activities were calculated from the linear portions of the reaction curve recorded over a period of 3 min at 237 nm. A change in absorbance value for complete hydrolysis of ATEE was taken as 400 M⁻¹. One unit of inhibition was defined as the quantity of inhibitor that reduces the rate of conversion of substrate by 1 μ mol/min under standard conditions of assay.

2.2.2. Purification of FBPI

2.2.2.1. Extraction. Soluble inhibitor was extracted from acetone-defatted flour of *Dolichos lablab perpureus* seeds by stirring a 10% suspension in 100 mM phosphate buffer, pH 7.6, for 4 hr at 4°C. The insoluble materials were removed by centrifugation at 9000 \times g for 30 min at 4°C. The pH of the supernatant was adjusted to 6.0 with 200 mM acetic acid, incubated at 60°C for 15 min in a water bath, and quenched in an ice bath. The precipitated proteins/nucleic acids were removed by centrifugation at 9000 \times g for 15 min. The pH of the supernatant was readjusted to 7.0 with 500 mM NH₄OH. This fraction was subjected to 60% (NH₄)₂SO₄ precipitation and kept overnight at 4°C. The precipitate was collected by centrifugation at 10,000 \times g, dissolved in a minimum amount of 100 mM phosphate buffer, pH 7.0, and dialyzed against 25 mM Tris-HCl buffer, pH 9.0, at 4°C. The dialyzate was centrifuged for 15 min at 10,000 \times g and the supernatant lyophilized.

2.2.2.2. Ion-Exchange Chromatography. One hundred thirty-five mg of lyophilized (NH₄)₂SO₄ fraction containing 80 mg of protein was dissolved in 10 ml of 50 mM Tris-HCl buffer, pH 9.0, containing 100 mM NaCl and applied onto a QAE-Sephadex column (1.5 \times 27 cm) equilibrated with the above buffer. The column was washed with two bed volumes of equilibration buffer, and bound proteins were eluted with a gradient of decreasing pH and increasing ionic strength (equilibration buffer and 100 mM phosphate buffer, pH 5.5, containing 400 mM NaCl). Fractions (4 ml each) were collected at a flow rate of 40 ml/hr, and those corresponding to trypsin and chymotrypsin inhibitory activities were pooled and lyophilized.

2.2.2.3. Gel Filtration. Twenty mg of QAE-Sephadex fraction in 1.3 ml of 100 mM phosphate buffer, pH 7.0, was applied onto a Sephadex G-75 column (1 \times 100 cm) equilibrated with the same buffer. The proteins were eluted by isocratic elution with the equilibration buffer. Fractions (1 ml each) were collected at a flow rate of 15 ml/hr, and those corresponding to trypsin and chymotrypsin inhibitory activities were pooled and lyophilized.

2.2.3. Characterization

2.2.3.1. Molecular Weight. Molecular weight was determined by SDS-PAGE, gel-permeation chromatography on a Sephadex G-75 column, and amino acid analysis.

2.2.3.2. Preparation of Antibodies and Immuno-diffusion. Polyclonal antibodies to FBPI were obtained by immunizing wild laying hens according to the method of Grassmann *et al.* (1990). A solution of FBPI (200 µg/100 µl) in PBS, pH 7.4, was emulsified with equal volume of Freund's complete adjuvant and was given subcutaneously (two sites) in pectoral muscle. Booster doses of the same concentration were given after 3 weeks. The collected eggs were marked, stored at 4°C, and partially pure IgY was obtained by PEG precipitation of yolk followed by DEAE-cellulose chromatography. Double immunodiffusion was performed essentially according to the method of Ouchterloney (1967).

2.2.3.3. Effect of Denaturing Agents. The stability of FBPI in the presence of 8 M urea was determined with a solution of 1 mg/ml in 8 M urea. The solution was kept at 10°C for 24 hr and diluted eight-fold with 100 mM phosphate buffer, pH 7.6, to obtain 1 M urea concentration. Aliquots corresponding to 10 µg of FBPI were used to determine the inhibitory activity. The effect of urea on enzyme was nullified by incorporating the same concentration of urea in the enzyme control. Similarly, the effect of guanidinium chloride on FBPI was tested with a 6 M solution.

2.2.3.4. Effect of Temperature. Inhibitor solutions of 150 µg/ml in 100 mM phosphate buffer, pH 7.6, were incubated at temperatures in the range of 30–97°C in a water bath for 10 min. The solutions were rapidly cooled in an ice bath and residual inhibitory activities were determined using appropriate aliquots at 37°C.

2.2.3.5. Effect of pH. The effect of pH was determined by incubating a 200 µg/ml solution of FBPI in appropriate buffers and incubating overnight at 4°C. The buffers used were citrate-phosphate (100 mM), pH 2.6, 3.2, 4.0; phosphate (100 mM), pH 7.6; glycine-NaOH (100 mM), pH 8.6, 9.6, and 10.0. Appropriate aliquots were used to quantify the inhibitory activities after diluting the solutions to 40 µg/ml with 100 mM phosphate buffer, pH 7.6.

2.2.3.6. Circular Dichroism Measurements. CD spectra were measured in an AVIV Model 62 HDS spectropolarimeter at 20°C using a cell of 0.5 cm path length. The inhibitor solution (3 mg/ml) in distilled water was used for scanning from 180 to 250 nm at intervals of 1 nm and dwell time of 3 sec. Spectra from an average of three consecutive scans were obtained.

2.2.3.7. Amino Acid Analysis. Amino acid composition of the FBPI was determined by the method of Spackman *et al.* (1958). Two mg each of dry protein sample was hydrolyzed with 6 M HCl in evacuated sealed tubes for 24, 48, and 72 hr at 110°C. The hydrolyzates were dried in a desiccator under vacuum on NaOH pellets. Dried hydrolyzates were dissolved in 2 ml of 100 mM citrate buffer, pH 6.0, and suitable aliquots were analyzed by an LKB-4400 amino acid analyzer equipped with computational data analyzer.

2.2.3.8. Isolation of Enzyme-Inhibitor Complexes. Complexes of trypsin-FBPI, chymotrypsin-FBPI, and ternary complex of trypsin-FBPI-chymotrypsin were isolated by gel-permeation chromatography on Sephadex G-75 under conditions identical to those employed in FBPI purification and molecular weight determination using 100 mM phosphate, pH 8.0.

2.2.3.9. Difference Spectra. Difference spectra during the complex formation were recorded by placing two pairs of matched cuvettes (1 cm) in tandem fashion in reference and sample compartments at 25°C. Reference cuvettes contained free enzyme and inhibitor solutions separately in 100 mM phosphate buffer, pH 7.6, while the sample cuvettes contained 1:1 mixture of enzyme and inhibitor solutions at the same concentrations as in the reference cuvettes.

2.2.3.10. Binding Stoichiometry and K_i . Stoichiometry of FBPI with trypsin and chymotrypsin was determined by activity titrations. Apparent dissociation constants were calculated from the plots of concentration of enzymes inhibited versus molar concentration of FBPI, according to the method of Grob (1950). Competition experiments were performed essentially according to the method of Feinstein and Feeney (1966) with catalytically inactive enzymes, TLCK-trypsin, and TPCK-chymotrypsin.

2.2.3.11. Modification of Reactive Site Amino Acids. Arginine residues were modified according to the method of Liu *et al.* (1968). Free amino groups of lysine were modified according to the method of Haynes and Feeney (1967) using 0.1% TNBS. Tyrosine residues were modified according to the method of Riordan and Velle (1972) using *N*-acetyl imidazole.

2.3. Other methods

2.3.1. Polyacrylamide Gel Electrophoresis (PAGE)

Discontinuous (nondissociating) PAGE was performed according to the method of Davis (1964). SDS-PAGE was carried out according to the method of Weber and Osborn (1969).

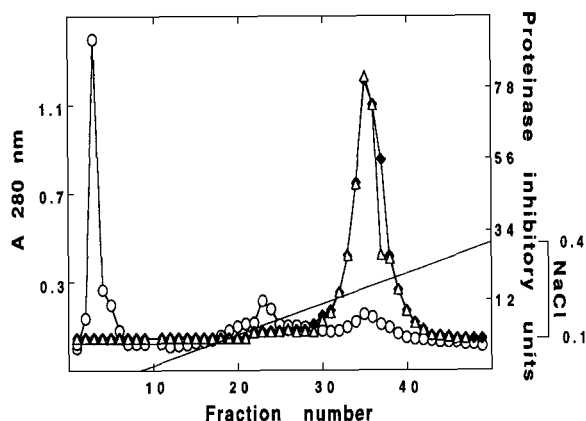


Fig. 1. Ion exchange chromatography of FBPI on a QAE-Sephadex column: trypsin inhibitory units (\blacklozenge); chymotrypsin inhibitory units (\triangle); A_{280} in nm (\circ). See Section 2 for details.

2.3.2. Staining Techniques

Proteinase inhibitory activity bands were visualized according to the method of Xavier-Filho and Moreira (1978). Staining for glycoproteins was carried out with PAS staining technique. Protein staining was carried out with 1% Coomassie brilliant blue R-250. Silver staining for proteins was carried out according to the method of Sammons *et al.* (1981).

2.3.3. Isoelectric Focusing

IEF was performed in 7.5% polyacrylamide gels containing 2% ampholyte carriers (Pharmacia) of pH 3–10 at 4°C. The gels were stained for protein and activity as indicated above. The ampholytes were leached out by soaking the gels in 10% trichloroacetic acid before staining for proteins.

2.3.4. Free Thiol Group and Protein Estimation

Free thiol groups were estimated using DTNB by the procedure of Habeeb (1972). Protein content for routine purpose, was determined by the method of Lowery *et al.* (1951), using BSA as standard.

3. RESULTS AND DISCUSSION

3.1. Purification

The major proteinase inhibitor has been purified and characterized from the lablab bean seeds with minimal loss of biological activity. The crude extract, when

subjected to electrophoresis and stained for inhibitory activity, exhibited a slow-moving minor inhibitor and a fast-moving major inhibitor. However, the seeds germinated for 24 hr showed an additional slow-moving species (data not shown). The possible explanation could be *de novo* synthesis of new inhibitor during germination (data not shown). Therefore, the dry seeds were used as a source for the native form of major inhibitor.

The proteins present in ammonium sulfate fraction (0–60% cut) were resolved using QAE-Sephadex ion-exchange chromatography (Fig. 1). The inhibitory activity eluted as a single peak, and the fractions containing activity were pooled, dialyzed, and lyophilized. The QAE-Sephadex fraction so obtained was still heterogeneous, containing minor noninhibitory proteins, which were removed by gel-permeation chromatography on Sephadex G-75. The final preparation was homogeneous by PAGE, SDS-PAGE (Fig. 2), and double immunodiffusion (data not shown). The recoveries and relative fold purification at different stages of purification are given in Table I. During the different stages of purification, the ratio of trypsin-inhibitory activity to chymotrypsin-inhibitory activity remained nearly constant. This copurification of these two inhibitory activities indicated that both the activities may reside on the same protein. Such double-headed inhibitors have been reported from horsegram (Ramasarma and Rajagopal Rao, 1991) and most of the plants which have Bowman-Birk inhibitors (Laskowski and Kato, 1980).

3.2. Characterization

Lack of reports pertinent to molecular properties of proteinase inhibitor from this major legume led us to study the molecular properties of the purified inhibitor. The molecular weight of the purified preparation was estimated to be $18,500 \pm 100$ Da by SDS-PAGE, amino acid analysis, and gel-permeation chromatography (Fig. 3). FBPI with a M_r of 18.5 kDa closely resembled many legume inhibitors such as double-headed inhibitors from pigeon pea (Oswole *et al.*, 1992), *E. latissima*, great northern bean (Rayas-Duarte *et al.*, 1992), navy bean (Norton, 1991), and *Psophocarpus tetragonolobus* (Richardson, 1991). The concentration-dependent dimerization exhibited by a few proteinase inhibitors was not observed in the case of FBPI. Though FBPI is a double-headed inhibitor with higher cysteine content, it differed from Bowman-Birk inhibitors in having a relatively high molecular weight.

The FBPI exhibited an isoelectric pH of 4.3, which is comparable to those of well-characterized inhibitors

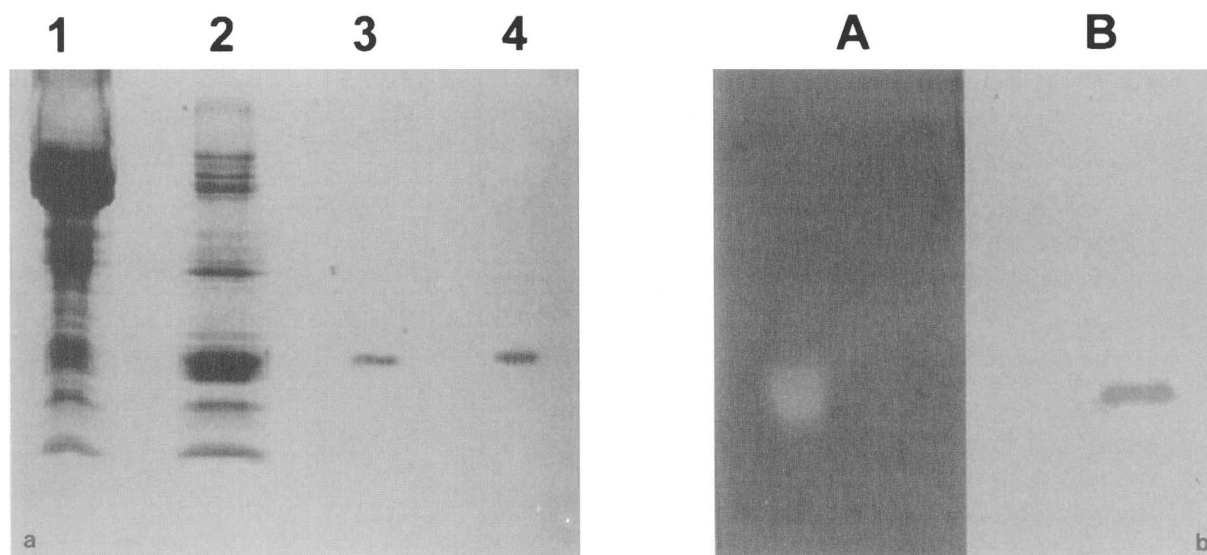


Fig. 2. (a) SDS-PAGE of FBPI at different stages of purification; loaded onto the gel were (lane 1) 250 µg of crude protein, (lane 2) 150 µg of 0–60% ammonium sulfate fractionate (lane 3) 25 µg of QAE-Sephadex fraction, (lane 4) 25 µg of Sephadex G-75 preparation. (b) Native PAGE of the purified FBPI; lane A, 10 µg of FBPI stained for trypsin-inhibitory activity; lane B, 10 µg of FBPI stained for protein by silver stain. See Section 2 for details.

Table I. Purification of FBPI from 100-g Seeds of *Dolichos lablab perpureus* L. cv-HA-3^a

Purification step	Total protein (mg)	Total activity		Specific activity		Percent yield		Fold purification	
		TIU × 10 ³	CIU × 10 ³	TIU/mg	CIU/mg	TIA	CIA	TIA	CIA
Crude extract	19,300	13487.5	3431.5	698.8	177.8	100	100	1.0	1.0
pH fractionate	9,680	13217.7	2831.2	1365.4	292.5	98.0	82.5	2.0	1.7
(NH ₄) ₂ SO ₄ fraction	2,700	11980.0	2431.4	4437.0	900.5	88.8	70.8	6.4	5.0
QAE-Sephadex	538	8735.8	2147.3	16237.5	3991.3	64.8	62.6	23.2	22.5
Sephadex G-75	344	6924.0	1917.8	20127.9	5575.0	51.3	55.9	28.8	31.4

^a Yield and fold purifications were calculated on the basis of trypsin inhibitory units (TIU) and chymotrypsin inhibitory units (CIU) using BAPNA and ATEE as substrates for trypsin and chymotrypsin, respectively. TIA, trypsin-inhibitory activity; CIA, chymotrypsin-inhibitory activity.

from the *Fabaceae* suborder and a few cereals (Las-kowski and Kato, 1980; Moosor *et al.*, 1984; Norton, 1991), with the exception of peanut inhibitor, which has a pI of 8.9. In general, proteinase inhibitors are stable under usual protein denaturing conditions. As expected, FBPI showed stability at acidic pH, high temperatures (25–90°C) both in acidic (4.0) and neutral (7.0) pH, 8 M urea, and 6 M guanidinium chloride. However, the protein was labile at alkaline pH (9.8–11.9). Although the thermostability of proteinase inhibitors including FBPI can be attributed to extensive cross-linking via disulfide bridges, it is hard to make a generalization, as inhibitors from cabbage foliage (Broadway, 1993) and

fingermillet (Manjunatha *et al.*, 1984), with only a few disulfide bridges, are also thermostable.

3.2.1. Amino Acid Composition

Amino acid composition data (Table II) indicate that the FBPI is rich in sulfur-containing amino acids and acidic amino acids (nearly 20%), comparable to those of well-known inhibitors of legumes and cereals (Norton, 1991). Basic amino acids, Lys, Arg, and His, were also present in considerable amount. The protein had a few aromatic amino acid residues, correlating with its low $E_{1\text{cm}}^{1\%}$ 280 nm (2.6). As in the case of inhibitors

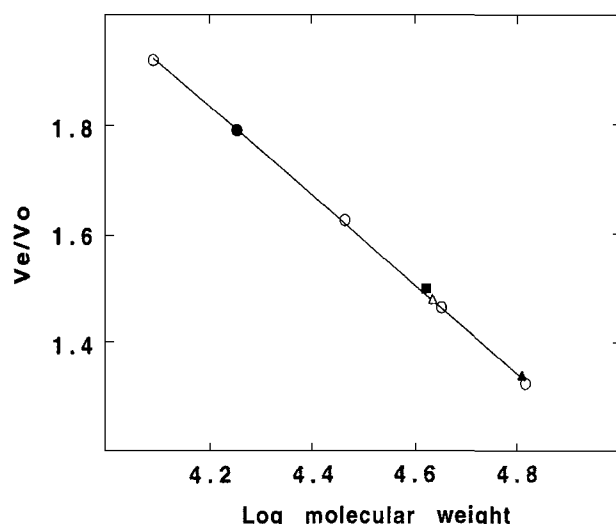


Fig. 3. Determination of molecular weights of FBPI and its complexes with trypsin and chymotrypsin by gel-permeation chromatography on a Sephadex G-75 column: molecular weight standards (○); FBPI (●); trypsin-FBPI complex (■); chymotrypsin-FBPI complex (□); ternary complex of FBPI-trypsin-chymotrypsin (▲). See Section 2 for details.

Table II. Amino Acid Composition of the FBPI^a

Amino acid	Residues/mole	Whole number
Ala	7.8	8.0
Asp/Asn	20.4	20.0
1/2-Cys	12.0	12.0
Arg	6.4	6.0
Glu/Gln	16.5	17.0
Gly	6.9	7.0
His	11.2	11.0
Ile	9.6	10.0
Leu	5.8	6.0
Lys	12.8	13.0
Met	2.1	2.0
Phe	5.0	5.0
Pro	12.9	13.0
Ser	14.9	15.0
Thr	12.1	12.0
Tyr	4.8	5.0
Val	2.0	2.0

^a Refer to Section 2 for further details.

of lima bean (Norton, 1991), ground nut (Ikenaka and Norioka, 1986), and horsegram (Ramasarma and Rajagopal Rao, 1991), the FBPI is also devoid of Trp. The absence of free thiol groups in FBPI despite a 12 mol % cysteine suggests the existence of disulfide bridges as observed in many BBIs.

3.2.2. Secondary Structure

The CD spectra of the FBPI in water did not show any minimum at 220 nm, suggesting a random coiled secondary structure. Similar observations were made with soybean BBI and chick pea inhibitors (Ikenaka and Norioka, 1986), although the presence of β -sheet has not been ruled out. The stability of FBPI to usual protein denaturants may be attributed to a lack of ordered secondary structures, namely α -helix and β -sheet. However, the thermostable chymotrypsin inhibitor from maize (Shulmina *et al.*, 1985) and trypsin inhibitors from squash (Hilder *et al.*, 1987) and ridged gourd (Halder *et al.*, 1996) are unique with α -helical content. Interestingly, the maize inhibitor exhibited stability at a pH range of 3–10. Generally, the α -helix and β -sheet secondary structures confer lability to denaturing agents such as temperature and pH. However, the thermolabile STI, which lacks α -helix and β -sheet, and thermostable inhibitors from barley and finger millet, which contain α -helix and β -sheet (Richardson, 1991), indicate that the secondary structures are not the sole determinants of protein stability.

3.2.3. Specificity

The purified FBPI exhibited inhibitory activity against serine proteinases, and was inactive against cysteine and aspartic proteinases. Within this class, the most preferred enzyme was trypsin. Such varied specificities are commonly found in legume and nonlegume inhibitors (Norton, 1991; Ikenaka and Norioka, 1986). The inhibitor also exhibited species-specific inhibition of the proteinases, i.e., its potency to inhibit bovine trypsin was more pronounced than the porcine trypsin. Such preferences have been exhibited by cereal inhibitors of sorghum and finger millet (Manjunatha *et al.*, 1984). In addition to species-specific inhibition, few cereal inhibitors inhibit enzymes from two different classes, namely trypsin and amylase (Manjunatha *et al.*, 1984; Richardson, 1991). However, the FBPI did not show any α -amylase inhibitory activity.

3.2.4. Stoichiometry and K_i

Kinetic studies with FBPI showed competitive inhibition for both trypsin and chymotrypsin. Inhibition in the presence of synthetic ester substrates BAPNA and ATEE for trypsin and chymotrypsin, respectively, gave an enzyme to inhibitor stoichiometry of 1:1. On the other hand, inhibition in the presence of casein indicated a

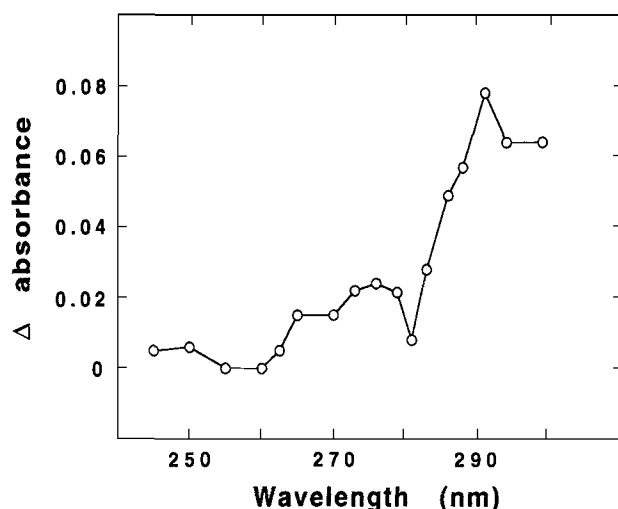


Fig. 4. Difference spectrum of FBPI-trypsin complex against FBPI and trypsin at an individual concentrations of 4×10^{-5} M in 100 mM phosphate buffer, pH 7.6; spectrum is an average of three recordings.

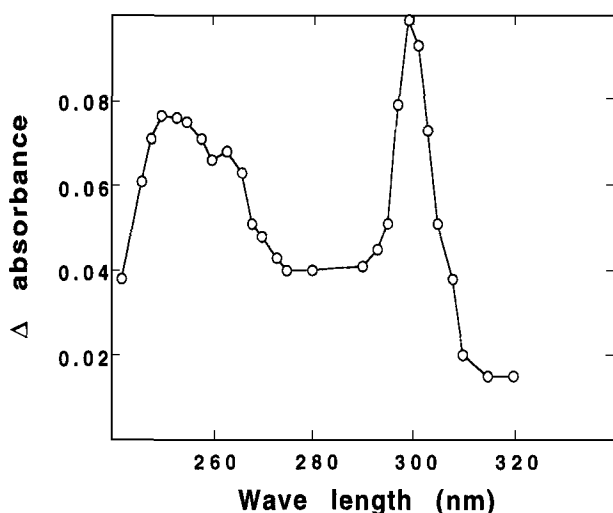


Fig. 5. Difference spectrum of FBPI-chymotrypsin complex against FBPI and chymotrypsin at individual concentrations of 4×10^{-5} M in 100 mM phosphate buffer, pH 7.6; spectrum is an average of three recordings.

stoichiometry of 1:2. The complexes of trypsin and chymotrypsin with FBPI were isolated using Sephadex G-75. The molecular weights of the complexes corresponded to additive molecular weights of FBPI and enzymes, indicating a 1:1 molar stoichiometry. In another set of experiments, FBPI-enzyme complexes of trypsin and chymotrypsin were incubated with molar excess of free enzymes and were subjected to gel permeation chromatography. The results indicated the formation of a ter-

nary complex of FBPI with trypsin and chymotrypsin. The above results, and the fact that even a tenfold excess of chymotrypsin (as evidenced by competition assay) could relieve only 50% of trypsin inhibition, indicated that these two enzymes are independently inhibited by FBPI.

The dissociation constants for trypsin and chymotrypsin-FBPI complexes were 2.1 and 3.1×10^7 M $^{-1}$, respectively. The values were similar to those reported for general proteinase-inhibitor interactions, 1×10^8 – 12×10^8 M $^{-1}$ (Norton, 1991). Further, these values also indicated that trypsin is the preferred enzyme for FBPI inhibition over chymotrypsin. These kinetic constants and the fact that the FBPI competitively inhibited trypsin and chymotrypsin suggest that FBPI-trypsin/chymotrypsin interactions involve the standard mechanism of proteinase-proteinase inhibitor interaction. Interaction of trypsin and chymotrypsin with FBPI was also studied by difference spectral analysis. Trypsin and FBPI at individual concentration of 4×10^{-5} M each showed a difference spectrum with a minor peak of absorption at 275 nm, a shoulder from 287 to 290 nm due to tyrosine of either of the partners, and a major peak at 295 nm, exclusively due to tryptophan of trypsin, as the FBPI lacks Trp (Fig. 4). Similarly, the difference spectrum of chymotrypsin-FBPI interactions showed a peak at 250 nm due to phenylalanine of either of the partners, and peak II at 295 nm due to tryptophan of chymotrypsin (Fig. 5). Thus, the difference spectra indicated molecular rearrangements both in enzyme and inhibitor molecules.

3.2.5. Reactive Sites

A few amino acid residues of the purified FBPI were modified in order to gain some preliminary insight concerning the reactive-site amino acids essential for inhibition of trypsin and chymotrypsin. The rate of modification of Lys in the first 12 min was commensurate with that of loss in trypsin inhibitory activity, indicating the presence of a fast-reacting amino group. Although loss was not commensurate after 70 min, complete loss of trypsin inhibitory activity was observed after 24 hr with no effect on chymotrypsin inhibitory activity. The modification of arginine did not affect the activity of FBPI toward either of the enzymes. However, tyrosine modification greatly affected the chymotrypsin inhibitory activity of FBPI. These results indicated the involvement of Lys and Tyr in the reactive sites of FBPI for trypsin and chymotrypsin, respectively. Further, the presence of Lys in the reactive site for trypsin suggested

that it is a Lys-type trypsin inhibitor (Laskowski and Kato, 1980; Laskowski, 1986). Independent binding of trypsin and chymotrypsin by FBPI as evidenced by competition and modification studies suggested that it is a double-headed inhibitor like many legume inhibitors (Laskowski, 1986). Reports pertaining to the essentiality of active serine proteinase for proteinase-inhibitor interaction are inconclusive. The results of competition experiments with FBPI indicated that the inhibitor has higher affinity toward catalytically active enzymes.

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